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TITLE: Role of CREB in CML

PRINCIPAL INVESTIGATOR: Kathleen M. Sakamoto, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California
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6. AUTHOR(S)

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5e. TASK NUMBER

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14. ABSTRACT

The purpose of this proposal is to understand the molecular pathways regulating Bcr-Abl positive CML cells. We demonstrated that the transcription factor, CREB, is highly expressed in K562 cells and cells from patients with chronic phase CML. This led us to hypothesize that CREB may play a critical role in regulating proliferation of CML cells. To determine whether CREB and CREB-dependent pathways may be bonafide targets for CML therapy, we chose to downregulate CREB using RNA interference. There are two specific aims. In Aim 1, we will test the hypothesis that downregulation of CREB inhibits proliferation and survival of CML cells. In Aim 2, will test the hypothesis that downregulation of CREB inhibits leukemia progression in vivo and in primary CML cells. We have generated CREB shRNA lentivirus and infected primary mouse and human bone marrow stem cells. We have also infected Ba/F3 cells expressing the T315I mutation of Bcr-Abl with and without CREB shRNA and followed leukemia progression in vivo. Our results suggest that CREB is necessary for both normal stem cell proliferation and differentiation, and leukemic progression.

15. SUBJECT TERMS
CREB, RNA interference, CML, signaling

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INTRODUCTION

CREB is a transcription factor that regulates proliferation and survival in both hematopoietic and neuronal cells. We found that overexpressed in the blood and bone marrow from patients with chronic phase CML. We hypothesize that CREB and CREB-dependent signaling molecules may be effective targets for CML therapy. To test this hypothesis we have chosen to downregulate CREB using RNA interference.

BODY

Statement of Work

Task 1. To test the hypothesis that downregulation of CREB will inhibit the proliferation and survival of CML cells

These tasks have been accomplished and are following the timeline outlined in the proposal.

a. We have generated CREB shRNA constructs and generated lentivirus (Months 1 to 3).

b. We have infected various CML cell lines with lentivirus and characterize expression of CREB by Western Blot analysis and RT-PCR (Months 3 to 4).

c. We have tested the effects of CREB shRNA on cell proliferation, survival, and apoptosis (Months 4 to 10) and showed that CREB is necessary for proliferation and survival of K562 cells (CML cell line).

d. We have examined CREB downstream gene expression, using microarray analysis (Months 10 to 12) and have identified 200 genes that are significantly upregulated and downregulated that also have CREB binding sites according to “chip on chip” data published by Marc Montminy.

Task 2. To test the hypothesis that downregulation of CREB inhibits leukemia progression *in vivo* and in primary CML cells

a. We have generated CREB shRNA, bcr-abl, and control retrovirus (Months 1 to 3).

b. We have examined the effects of CREB shRNA retroviral infection on normal stem cells and follow mice for engraftment in bone marrow transplantation assays (Months 3 to 12). We have also shown that primary hematopoietic stem cells require CREB for proliferation and differentiation *in vitro*.

c. We will infect mouse bone marrow with CREB and bcr-abl, or control shRNA retrovirus and perform bone marrow transplantation assays. Latency and type of leukemia will be characterized (Months 12 to 24).

These experiments are pending. We have also infected Ba/F3 cells containing the T315I mutation that also express the luciferase gene and injected these cells into SCID mice. Ba/F3 T315I mutation cells infected with CREB shRNA have delayed progression of leukemia compared to scrambled shRNA controls.

d. Experiments yet to do: We will infect primary human CML stem cells with CREB shRNA and follow proliferation in methylcellulose (3 to 12 months).

e. If time permits, we will transplant primary CML stem cells transduced with CREB shRNA into NOD/SCID mice and follow mice for development of leukemia (6 to 24 months).

So far, our results are novel and have not been previously described.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration that CREB is required for normal hematopoietic stem cell proliferation and differentiation.
2. Demonstration that CREB is necessary for CML cell proliferation and survival.
3. Demonstration that CREB inhibits leukemia progression of resistant Bcr-Abl cells in vivo.

REPORTABLE OUTCOMES

Papers:

1. Cheng JC and KM Sakamoto. Novel Technologies in Stem Cells: RNA interference and Stem Cells. Stem Cells, *in press*.

2. Cheng JC, Kinjo K, Wu W, Schmid I, Shankar DB, Stripecke R, Kasahara N, Pellegrini M, Nelson S, and KM Sakamoto. The Requirement for CREB in Normal Hematopoiesis and Leukemogenesis. Manuscript in preparation.

Abstracts:

1. KM Sakamoto. Requirement of CREB in normal myelopoiesis and leukemogenesis. Presentation at Myeloid Workshop, American Society for Hematology, Orlando FL, December 2006.

2. Cheng JC, Shankar D, and KM Sakamoto. Requirement of CREB in Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

CONCLUSIONS

Our results suggest that CREB plays a critical role in normal cells and CML cells. We are continuing to validate the requirement for CREB in primary

CML cells *in vitro* and *in vivo*. These are novel findings and will advance our understanding of normal and malignant hematopoiesis.

REFERENCES: Not applicable.

APPENDICES: Curriculum Vitae and Manuscript by Cheng and Sakamoto, *in press.*

SUPPORTING DATA: See below.

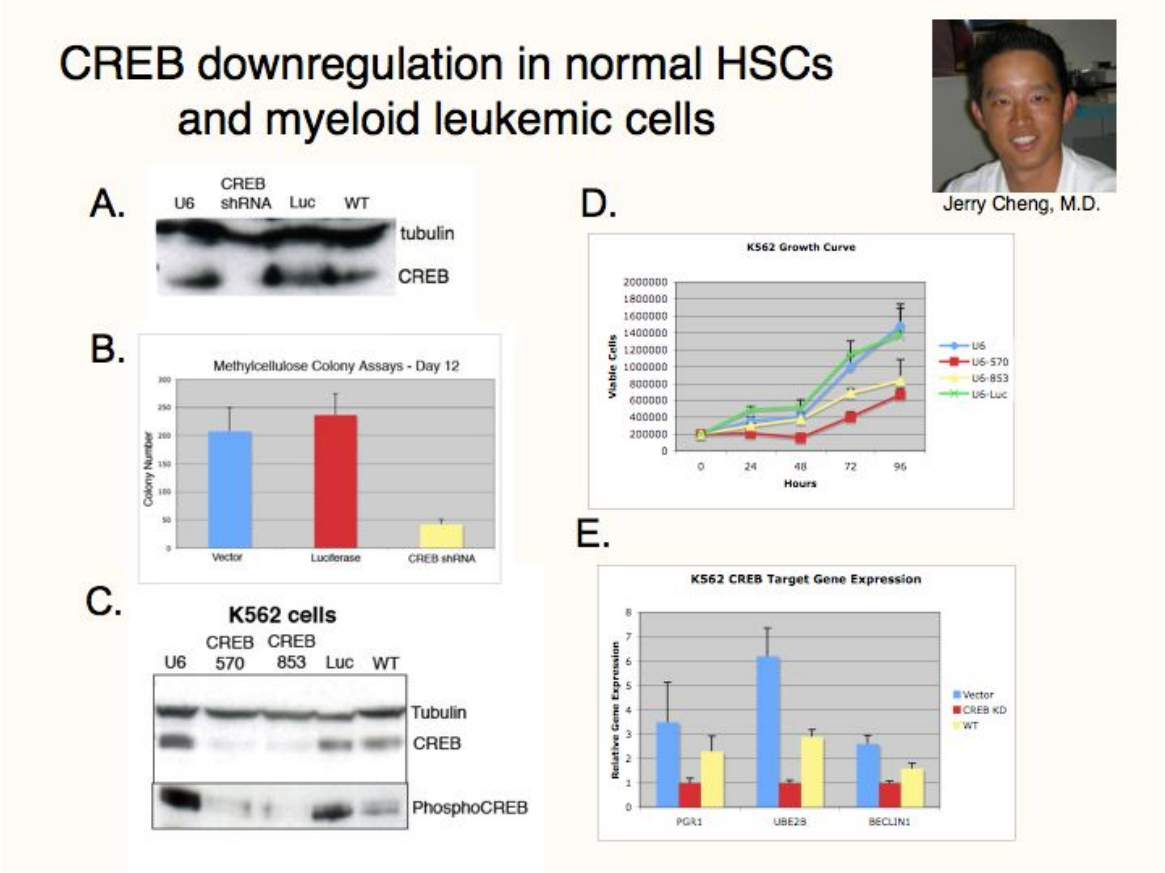

Figure 1. CREB downregulation in normal hematopoietic stem cells (HSC) and CML cells. (A) CREB shRNA lentivirus induces downregulation of CREB by >80%. (B) CREB downregulation results in decreased proliferation and differentiation of normal HSCs. (C) CREB is downregulated in K562 cells infected with CREB shRNA lentivirus. (D) CREB shRNA suppresses K562 proliferation. (E) Candidate target genes in CREB shRNA transduced K562 cells identified using microarray analysis.

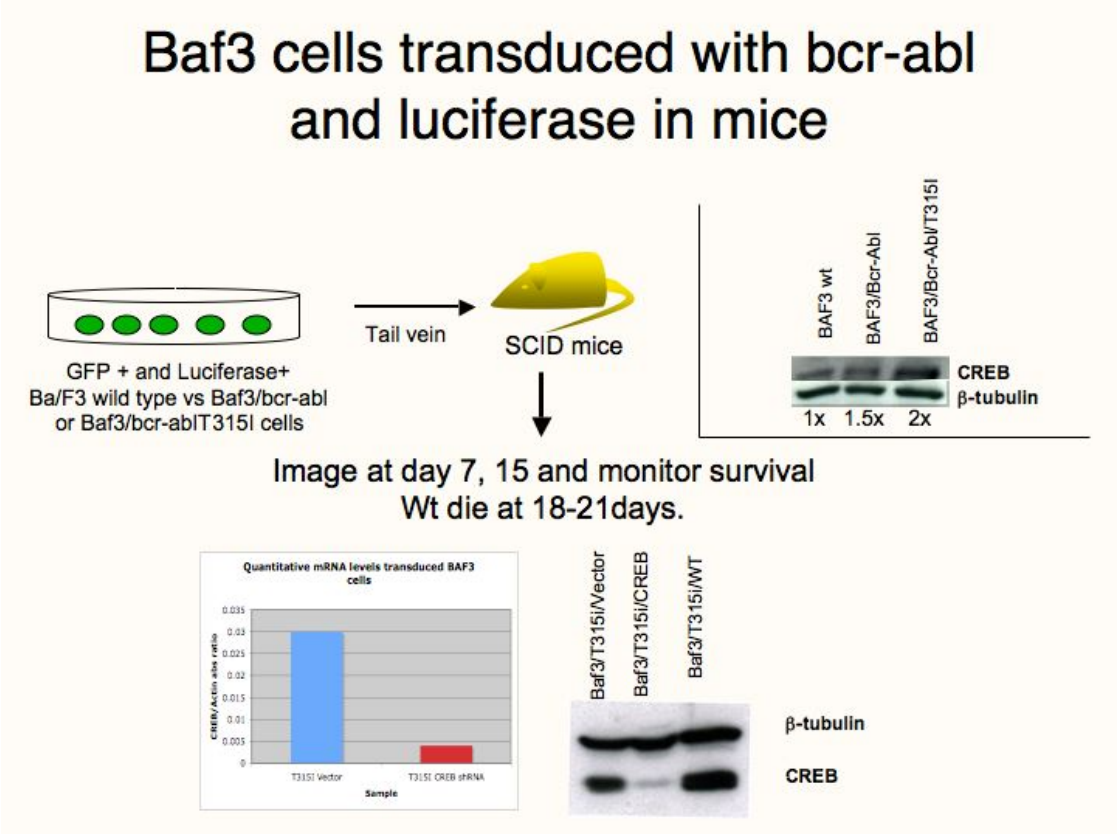

Figure 2. Ba/F3 cells expressing Bcr-Abl T315I mutation. (upper right) CREB is overexpressed in Ba/F3 cells overexpressing Bcr-Abl T315I (Gleevec resistant) mutation by Western Blot analysis. CREB shRNA transduced Ba/F3 cells have significant decrease in CREB expression by quantitative RT-PCR (lower left) and Western blot analysis (lower right).

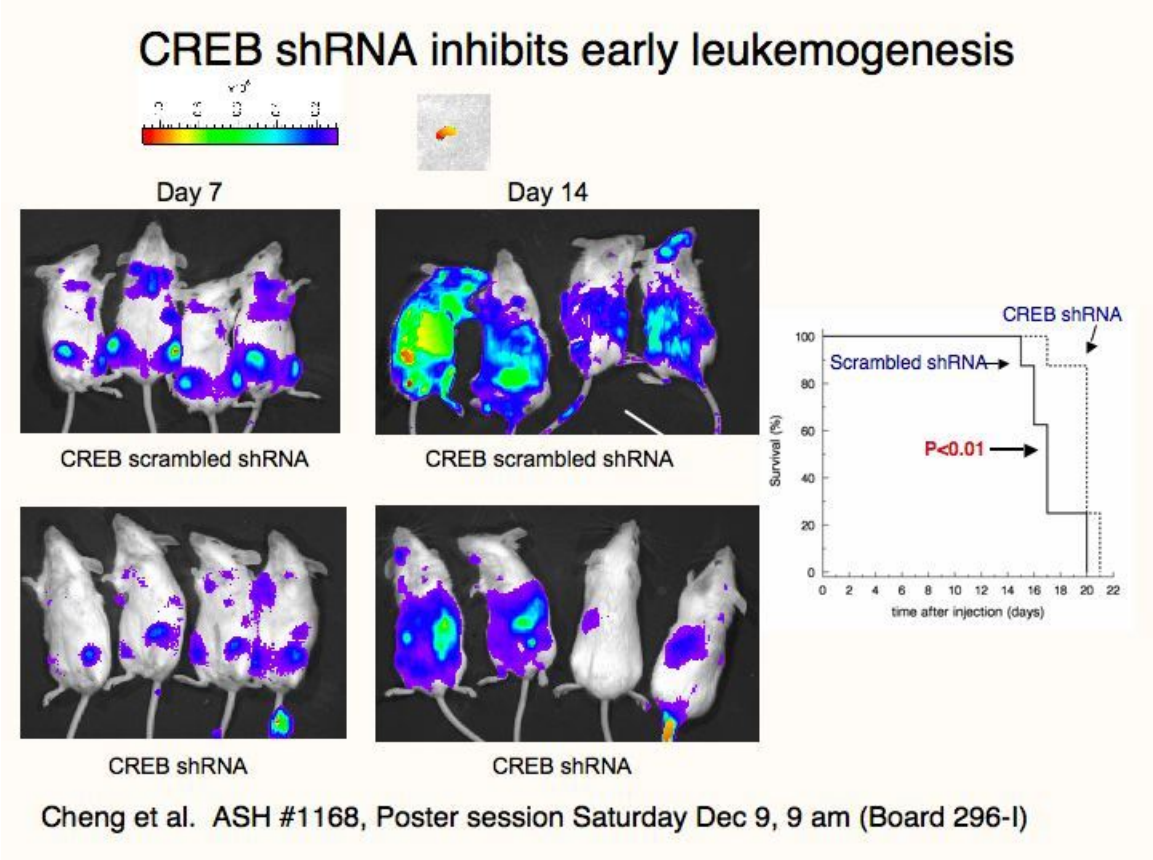

Figure 3. CREB shRNA inhibits early leukemogenesis. (left) Ba/F3 cells that express T315I mutation and luciferase, and transduced with either CREB or scrambled shRNA lentivirus were injected into the tails of SCID mice. Disease progression was followed by bioluminescent imaging. (right) CREB shRNA resulted in a significant decrease in 50% survival of injected mice ($p<0.01$).

CURRICULUM VITAE

KATHLEEN MIHO SAKAMOTO, M.D., Ph.D.

CURRENT POSITION
Vice-Chair, Translational Research
Professor and Chief
Division of Hematology-Oncology
Mattel Children's Hospital
Department of Pediatrics and
Department of Pathology and Laboratory Medicine
David Geffen School of Medicine at UCLA

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CITIZENSHIP
United States

EDUCATION

1975-1979	Williams College, Williamstown, MA; B.A. Cum Laude; Biology
1981-1985	University of Cincinnati, College of Medicine, Cincinnati, OH; M.D.
2000-2003	California Institute of Technology, Pasadena, CA; Ph.D. Biology Howard Hughes Medical Institute, Laboratory of Raymond J. Deshaies

LICENSURE

1986 California License, G58328
1994 DEA license, BS1361992

BOARD CERTIFICATION

1986 Diplomate, National Board of Medical Examiners
1989 Diplomate, American Board of Pediatrics (recertified 1999)
1994 Diplomate, American Board of Pediatrics, Hematology-Oncology (recertified 1999)

RESEARCH INTERESTS

Growth Factor Signal Transduction
Leukemogenesis
Cell Cycle Regulation and Hematopoiesis

EMPLOYMENT

1985-1986	Internship, Pediatrics, Children's Hospital of Los Angeles
1986-1988	Residency, Pediatrics, Children's Hospital of Los Angeles
1988-1989	Fellowship, Hematology/Oncology, Children's Hospital of Los Angeles
1991-1993	Clinical Instructor, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1993-1994	Visiting Assistant Professor of Pediatrics, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1994-1998	Assistant Professor of Pediatrics, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1995-present	Joint appointment, Department of Pathology and Laboratory Medicine
1998-present	Associate Professor of Pediatrics and Pathology, Mattel Children's Hospital at UCLA, UCLA School of Medicine
2003-present	Professor of Pediatrics and Pathology & Laboratory Medicine
2004-present	Visiting Associate, Division of Biology, California Institute of Technology
2005-present	Professor of Pediatrics and Pathology & Laboratory Medicine, David Geffen School of Medicine at UCLA
2005-present	Chief of Division of Hematology-Oncology, Mattel Children's Hospital, David Geffen School of Medicine at UCLA
2006-present	Vice-Chair of Translational Research, Mattel Children's Hospital, David Geffen School of Medicine at UCLA

RESEARCH EXPERIENCE

1978-1979	Senior Honors Thesis, Department of Biology, Williams College. "Effects of Centrifugation Time on Separation of Plant Organelles".
1979-1980	Research Assistant, Department of Biochemical Genetics, City of Hope Medical Center
1993-1996	Research Assistant, Department of Physiology, USC School of Medicine,
1980-1991	Postdoctoral Fellow, Division of Hematology-Oncology, in the laboratory of Judith C. Gasson, Ph.D., UCLA School of Medicine
1999	Visiting Associate, laboratory of Raymond Deshaies, Ph.D., Department of Biology, California Institute of Technology, Pasadena, CA
2000-2003	STAR program/Graduate Studies, Division of Biology, Laboratory of Raymond Deshaies, PhD, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA

HONORS

1988	Victor E. Stork Award, Children's Hospital of Los Angeles
1990-1993	Leukemia Society of America Fellowship Award
1991	Leukemia Society of America award as First Designated Researcher supported by the Leukemia Society staff
1992-1995	Jonsson Comprehensive Cancer Center/STOP CANCER Career Development Award
1996-2002	Leukemia Society of America Special Fellow Award
1994	Young Investigator Award in Oncology, American Society for Pediatric Hematology-Oncology
1995	UCLA Frontiers of Science Award
1996	Ross Award in Research By Young Investigators (Western Society for Pediatric Research)

1998-2003	Leukemia Society of America Scholar Award
1998	Elected Council Member, Western Society for Pediatric Research
1998	Participant, AAMC Workshop for Senior Women in Academic Medicine
1999	Invited Participant, American Cancer Society Professors Meeting, October, New York
1997	"Meet-the-Expert", Signal Transduction and Cell Cycle Control in Myeloid Cells, American Society of Hematology, New Orleans, LA
1998	Katherine E. Rogers Scholar for Excellence in Cancer Research, Jonsson Comprehensive Cancer Center, UCLA
1999	Member of Scientific Review Committee, CONCERN Foundation
2000	AACR-Novartis Scholar in Training Award, Oncogenomics meeting, Tucson, AZ
2001	Keystone Symposium on "Cell Cycle" - Travel Award, Keystone, CO.
2002	AACR-AFLAC Scholar-in-Training Award, meeting on Ubiquitination and Cancer meeting, Vancouver, Canada.
2002	Full member, Molecular Biology Institute, UCLA
2003-present	Children's Oncology Group, Myeloid Biology Subcommittee
2003-2007	Member, Grant Review Subcommittee on Leukemia, Immunology, and Blood Cell Development for American Cancer Society
2004	NIH Study Sections on Drug Discovery and Molecular Pharmacology and Basic Mechanisms of Cancer Therapy, and Special Emphasis Panel on Diamond-Blackfan Anemia and Bone Marrow Failure syndromes
2004	Grant Reviewer, UC Discovery Biotechnology Program
2004	Moderator, Leukemia Session at American Society for Pediatric Hematology-Oncology Annual Meeting
2004	Abstract Reviewer and Moderator for "Hematopoiesis: Regulation of Gene Transcription," ASH Meeting
2005-2009	Member, NIH Hematopoiesis Study Section
2004	Grant Reviewer, Susan G. Komen Breast Cancer Foundation
2005	"Ask the Experts" in Pediatric Cancer, AACR Public Forum, Anaheim, CA.
2005	Chair of Minisymposium, Modulation of Protein Stability. AACR, Anaheim, CA.
2005-present	Member, Translation Research Program Review Subcommittee for the Leukemia and Lymphoma Society of America
2005	Moderator, Pediatric Hematology-Oncology session, PAS/ASPHO meeting, Washington DC, May 2005
2005-2009	Grant Reviewer, California Research Cancer Committee (CRCC)
2005-2009	Member, ASH Scientific Committee on Myeloid Biology
2006	Reviewer, NIH Oncology Postdoctoral Fellowship Committee
2006-present	CDMRP (DOD) CML Grant Review Committee
2006	ASH abstract reviewer on "Hematopoiesis: Regulation of Gene Transcription" for annual meeting
2006	UCLA Finalist, Margaret Early Trust Award
2006	Benjamin Franklin High School Wall of Fame Award
2006	Member, American Pediatric Society
2007-2008	Chairman, ASH Myeloid Biology Subcommittee

EDITORIAL BOARD/REVIEWER

Editorial board: Stem Cells

Medical Editor, emedicine online textbook for Pediatrics (Hematology-Oncology section)

Current Drugs, panel of evaluators

Ad hoc reviewer for journals: Ad hoc reviewer for journals: Blood, Oncogene, Proceedings of the National Academy of Sciences, Molecular and Cellular Biology, Journal of Cellular Biochemistry, Leukemia, Biotechniques, Cytometry, Pediatric Research, Cancer Research, Molecular Cancer Therapeutics, American Journal of Hematology, Molecular Genetics and Metabolism, American Journal of Human Genetics, New England Journal of Medicine, Pediatric Blood and Cancer, Cancer Research, Clinical Cancer Research, British Journal of Hematology, Clinical Prostate Cancer, Pediatrics

PROFESSIONAL SOCIETY MEMBERSHIPS

Candidate Fellow, American Academy of Pediatrics
Member, American Society of Hematology
Member, American Society of Pediatric Hematology-Oncology
Member, American Association for Cancer Research
Member, New York Academy of Science
Member, Western Society for Pediatric Research
Member, Society for Pediatric Research
Member, International Society for Experimental Hematology
Children's Oncology Group, AML Strategy Group
American Society for Biochemistry and Molecular Biology (ASBMB)
Member, ASPHO Meeting Committee
Member, ASPHO Career Development Task Force
Member, ASH Myeloid Biology Subcommittee

COMMITTEES

1994	Search Committee for Director of the Jonsson Cancer Center
1995	Search Committee for Nephrology Faculty Appointment
1996-1998	Admissions Committee, UCLA ACCESS program for graduate students
1996-1999	Admissions Committee, Medical Student Training Program, UCLA
1994-present	UCLA Cancer Committee
1998-1999	Chair of Tumor Cell Biology ACCESS Affinity Group for Graduate Students
1996-2002	Western Society for Pediatric Research (WSPR) Council member
2002-present	Search Committee for Pediatric Pulmonary
2002-present	Search Committee for Pediatric Nephrology
2002-present	Search Committee for Pediatric Hematology-Oncology
2006	Search Committee for Pediatric Cardiology
2006	Search Committee for Infectious Disease
2006	Committee for Loan Repayment, Department of Pediatrics
2006	Search Committee for Biostatistician, Department of Pediatrics
2006-present	Chair, Search Committee for Neonatology

2006-present Pediatric Credentials Committee

CAMPUS ACTIVITIES

1994-present Faculty Mentor on the Medical Student Training Program
1994-present Principal Investigator on the Tumor Cell Biology Training Grant
1995 Faculty Advisor Program for first year medical students
1995-present Principal Investigator on the UCLA ACCESS program for graduate Students

Teaching

1993-present Pediatric Hematology-Oncology elective
1993-present Advanced Clinical Clerkship in Pediatric Hematology-Oncology
1993-present Laboratory course in Biochemistry for first year medical students
1993-present Pediatric Clerkship
1993-present Advanced Clinical Clerkship in Pediatrics
1995 Ethics and Accountability in Biomedical Research
1995-1997 Major Concepts in Oncology
1995 Molecular and Cellular Foundations of Disease
1993-1997 Organization of Pediatric Hematology-Oncology weekly clinic conferences
1995-1999 Organization of the Pediatric Departmental Monthly Research Seminars
1999-2004 M229 Course on Cell Biology and Pathogenesis for ACCESS Graduate Students on "Cell Cycle" (organized by Patricia Johnson)
1996-2003 Pathophysiology Course in Hematopathology (session on Lymphoma)
2005-present Associate Director of the Signal Transduction Program Area, Jonsson Comprehensive Cancer Center
2005 MBI 298 seminar course on Ubiquitination
2005 Co-organizer, M294 Pathology course on Molecular Basis of Oncology

Clinical Activities

1993-present Medical Staff, Pediatric Hematology-Oncology, UCLA School of Medicine and Santa Monica Hospital

COMMUNITY SERVICE

Leukemia and Lymphoma Society of America, Los Angeles Chapter,Board of Trustees and Executive Board

PATENTS

"Proteolysis Targeting Chimeric Pharmaceutical" (Raymond Deshaies, Craig Crews, and Kathleen Sakamoto), Ref. No. CIT3284.

"RNA inhibition of CREB" (Jerry Cheng, Kathleen Sakamoto), UC Case No. 2003-348

GRANTS

1989-1990 American Cancer Society Clinical Oncology Fellowship

1990-1993 5 F32 CA08974-04 Individual National Research Service Award Molecular Analysis of Target Cell Response to Human GM-CSF ($102,100); National Cancer Institute (Judith Gasson, Ph.D., P.I.)

1996-2002 Fellowship Award, Molecular Characterization of GM-CSF Action ($70,000) Leukemia Society of America (Judith C. Gasson, Ph.D., P.I.)

1993-1998 K08 CA59463, Clinical Investigator Award, Molecular Characterization of GM-CSF Action ($383,400), National Cancer Institute (Judith Gasson, Ph.D. P.I.)

1993-1996 3017-93, Special Fellow Award, Molecular Analysis of GM-CSF Action ($100,400), Leukemia Society of America (K. Sakamoto, M.D., P.I.)

1992-1995 Career Development Award, Molecular Characterization of GM-CSF Action ($150,000), STOP CANCER (K. Sakamoto, M.D., P.I.)

1992-1993 Seed Grant, Mutation Analysis of Structure-Function Relationships of Human GM-CSF Receptor Beta Subunit ($30,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)

1992-1993 Mutation Analysis of Structure-Function Relationships of the Human GM-CSF Receptor Beta Subunit ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (declined) (K. Sakamoto, M.D., P.I.)

1993-1995 Molecular Regulation of egr-1 by IL-3 and PIXY321 in Myeloid Leukemias ($100,000), Concern II (K. Sakamoto, M.D., P.I.)

1994 The Role of Cyclins in Myeloid Leukemias ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (K. Sakamoto, P.I.)

1995 UCLA Academic Senate Award ($1,500), "Stem Cell Factor Activation of Signal Transduction in Myeloid Leukemic Cells" (K. Sakamoto, M.D., P.I.)

1995 UCLA Frontiers of Science Award, The Regulation and Functional Role of p55CDC in Myeloid Leukemias ($28,000) (K. Sakamoto, M.D., P.I.)

1995 UCLA Prime Faculty Research Award, Molecular Regulation of Myeloid Cell Differentiation ($25,000) (K. Sakamoto, M.D., P.I.)

1995 Seed Grant, The Role of SRE-Binding Proteins During Signal Transduction in Myeloid Leukemias ($27,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)

1995 New Assistant Professor Grant, Transcriptional Regulation of egr-1 by Stem Cell Factor in Myeloid Leukemias ($35,000), Cancer Research Coordinating Committee (K. Sakamoto, M.D., P.I.)

1995-1997	Shannon Award, NIH (NCI) 1R55CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($80,000) (K. Sakamoto, M.D., P.I.)
1996	Concern II Foundation; Molecular Analysis of IL-3 and PIXY321 Signaling Pathways in Myeloid Leukemias ($50,000) (K. Sakamoto, M.D., P.I.)
1996-2002	First Award R29CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($350,000), NIH/NCI (K. Sakamoto, M.D., P.I.)
7/97-6/99	UC Biotechnology STAR Project, S97-03 "p55Cdc and Cell Cycle Regulation" ($40,000); Amgen, Inc. and University of California (K. Sakamoto, M.D., P.I.)
7/98-6/99	Contract with Eli Lilly, Inc. "Multiple Resistance Genes in Leukemias" ($32, 000), Co-PI with Leonard Rome, Ph.D. (K. Sakamoto, M.D., P.I.)
7/98-6/99	Jonsson Comprehensive Cancer Center Seed Grant, “Use of Low Molecular Weight Heparin in Cancer Patients Receiving Stem Cell Transplants,” ($30,000), Co-P.I. with Dr. Sinisa Dovat, M.D. (fellow)
7/98-6/2003	Leukemia Society of America Scholar Award, 1497-99 "The Role of p55Cdc during Myelopoiesis" ($350,000), Leukemia Society of America (K. Sakamoto, P.I.)
1/99-12/2001	Investigator initiated grant, California Cancer Research Program, “Cell Cycle Control and Cancer” ($400,000), California Department of Health Services (K. Sakamoto, P.I.)
7/99- 6/2000	Jonsson Comprehensive Cancer Center Seed Grant, “Development of a Novel Class of Protein-inhibiting Anti-cancer Therapeutics” ($15,000), K. Sakamoto (P.I.) and Raymond Deshaies (Co-P.I., Caltech)
1/2000	CaPCURE research award, “Development of a Novel Class of Protein-Inhibiting Therapeutics for Prostate Cancer” ($100,000). Raymond Deshaies (P.I., Caltech), K. Sakamoto, and Craig Crews (Co-P.I., Yale University).
1/99-12/02	Research Project Grant, "Molecular Analysis of Myeloid Cell Proliferation" ($300,000); American Cancer Society (K. Sakamoto, P.I.)
8/01-7/03	UC Biostar, “Targeting the estrogen receptor for Proteolysis”, with Celgene, Inc. ($40,000), K Sakamoto, P.I.
1/02-12/02	CaPCURE research award, “Targeting the Androgen Receptor for Degradation in Prostate Cancer” ($75,000) K.Sakamoto (P.I.), Raymond Deshaies (Co-P.I., Caltech) and Craig Crews (Co-P.I., Yale University).
6/02-7/03	National Cancer Coalition, “Signal Transduction and Cell Cycle Analysis in Leukemia” ($5,000), K. Sakamoto (P.I.).

1/03-12/06 American Cancer Society, Research Scholar Award. "The role of CREB in Leukemogenesis," ($625,000). K. Sakamoto (P.I.).

1/03-6/04 Department of Defense, "Targeting the estrogen receptor for ubiquitination and proteolysis in breast cancer," ($222,819). K. Sakamoto (P.I.)

1/03-12/03 Diamond-Blackfan Anemia Foundation, "AML in Diamond-Blackfan Anemia: Molecular Basis and Therapeutic Strategies," ($25,000). K. Sakamoto (P.I.)

1/1/03-12/31/04 SPORE grant in Prostate Cancer Research, Seed Grant Award, "Targeting the Androgen Receptor for proteolysis in Prostate Cancer," $75,000. K. Sakamoto (P.I.)

4/1/03-3/31/04 Stein-Oppenheimer Award, "Targeting the Estrogen Receptor in Breast Cancer," $20,000. K. Sakamoto (P.I.)

6/1/03-5/30/04 Genomic Exploration Seed Grant, Jonsson Comprehensive Cancer Center, "CREB and Human Leukemias," $5,000, K. Sakamoto (P.I.)

7/1/03-6/30/04 Susan G. Komen Breast Cancer Thesis Dissertation Award," $20,000. K. Sakamoto, R. J. Deshaies (P.I.)

1/04-12/07 NIH/NHLBI R01 (HL 75826), "The Role of CREB in Leukemogenesis," ($200,000/year). K. Sakamoto (P.I.)

9/04-8/06 R21, "Ubiquitination and Degradation in Cancer Therapy," ($135,000/year). K. Sakamoto (P.I.)

7/04-7/05 Department of Defense, "Identification of small non-peptidic ligands that bind the $SCF^{beta\text{-}TRCP}$ ubiquitin ligase to target the ER for ubiquitination and degradation ($75,000). K. Sakamoto (P.I.)

7/05-5/07 Fulbright Fellowship/MEC (Spain) postdoctoral fellowship, "Targeting the Androgen Receptor for Ubiquitination and Degradation: A new strategy for Therapy in Prostate Cancer" ($60,000), K. Sakamoto and R. Deshaies (Co-P.I.).

5/05 Boyer/Parvin Postdoctoral Fellow Award ($5,000), awarded to Deepa Shankar, Ph.D., K. Sakamoto (P.I.)

7/05 Stone Research Award ($1,000) award to undergraduate student Winston Wu, K. Sakamoto (P.I.)

7/05-6/07 Department of Defense postdoctoral fellowship, "Targeting the Androgen Receptor for Ubiquitination and Degradation: A New Strategy for Therapy in Prostate Cancer," ($80,000), K. Sakamoto (P.I.)

10/05-9/05 Diamond Blackfan Anemia Foundation, " Developing a zebrafish model of Diamond Blackfan Anemia." $25,000 (K. Sakamoto and S. Lin, P.I.)

10/05-9/09 NIH/NHLBI R01 (HL083077), " Molecular and Cellular Characterization of MPD." $225,000/ year (K. Sakamoto, P.I.).

7/06-6/08 Department of Defense, "The Role of CREB in CML," $45,800/year (K. Sakamoto, P.I.).

7/06-6/08 F32 HL085013 NRSA (NHLBI), "CREB and Hematopoietic Stem Cells," awarded to postdoctoral fellow Jerry Cheng, M.D. (K. Sakamoto, P.I.).

7/06-6/08 NCI T32 CA09056 Tumor Cell Biology Training Grant, "Studies in the Mechanisms of Targeted Therapy for Acute Myeloid Leukemia," for Alan K. Ikeda, M.D. (K. Sakamoto, P.I.).

10/06-9/09 Leukemia and Lymphoma Society Translational Research Grant, "Targeting Signaling Pathways in Pediatric AML." $200,000/year (K. Sakamoto, P.I. and Ted Moore, co-P.I.).

1/07-12/12 NHLBI, "Training in Developmental Hematology." $225,000/year (K. Sakamoto, P.I.) [Score 153, waiting for funding decision].

TRAINING FACULTY ON THE FOLLOWING TRAINING GRANTS (NIH T32 and K12 Programs)

Tumor Cell Biology
Tumor Immunology
Human and Molecular Development
Hematology
Vascular Biology
Neonatology
Medical Scientist Training Program (MSTP)
Gene Medicine
Stem Cell Research Institute

TRAINEES

1991-1993	Hu-Jung Julie Lee, undergraduate student
1992-1993	Elana Lehman, medical student
1993-present	Kathy Hwain Shin, undergraduate student, Work/study and Lab Assistant
1994-1995	Robert C. Mignacca, M.D., postdoctoral fellow
1994-1995	Stephen Phillips, undergraduate student, Student Research Project
1995	Allison Wong, medical student; Short Term Training Program; Recipient
of	Howard Hughes NIH Research Scholar Award, 1996-1997
1995	Ramona Rodriguez, medical student; Short Term Training Program,
	Centers of Excellence
1995-2000	Evelyn Kwon, graduate student
1996	Michael Mendoza, medical student, Short Term Training Program;
Centers	of Excellence and FIRST/STAR Award recipient
1996-2002	Patricia Mora-Garcia (awarded Minority Supplement Award from
	NIH/NCI), Dept. Pathology and Laboratory Medicine

1996-2002	Michael Lin, graduate students (recipient of NIH/NCI Tumor Cell Biology Training Grant), Dept. Pathology and Laboratory Medicine
1997	Raymond Wang, medical student, Short Term Training Program
1995-1999	Wayne Chu, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of 1999 Merle Carson Lectureship, 1st Prize Southwestern Pediatric Society, The Tenth Joseph St. Geme, Jr. Research Award for UCLA Pediatric Trainees)
1999-2000	Kristin Baird, M.D. Pediatric Resident, Mattel Children's Hospital at UCLA, research elective
2000-present	Deepa Shankar, Ph.D., Postdoctoral fellow (NIH Tumor Cell Biology Postdoctoral fellowship, JCCC fellowship).
2001-2002	Heather Crans, graduate student (recipient of NIH Tumor Immunology Training Grant), Dept. Pathology and Laboratory Medicine
2001-2003	Athena Countouriotis, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of Resident Research Award, American Academy of Pediatrics)
2002-present	Jerry Cheng, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA (won SPR House Officer Award 2003, ASPHO/SPR meeting, Seattle, WA).
2002-2003	Tamara Greene, Medical Student, UCLA School of Medicine
2002-2003	Johnny Chang, M.D., Medical Oncology Fellow, Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine (recipient Of NIH Hematology Training Grant)
2003-present	Noah Federman, M.D., Pediatric Resident, Mattel Children's Hospital, research elective
2003	Andy Liu, undergraduate student (Recipient of Undergraduate scholarship award for research performed in my laboratory)
2003	Ryan Stevenson, undergraduate student (now in medical school)
2004	Maricela Rodriguez, medical student
2005	Jenny Hernandez, Saul Priceman, Jose Cordero, Gloria Gonzales, Salemiz Sandoval
2005	Cid Sumolong, STTP, UCLA medical student
2005-2006	Winston Wu, undergraduate (recipient of John Stone Award for research performed in my laboratory)
2005-present	Salemiz Sandoval, graduate student (MBI)
2005-present	Samuel Esparza, M.D., Pediatric Hematology-Oncology fellow, STAR/PhD graduate program
2005-present	Jerry Cheng, M.D., Pediatric Hematology-Oncology fellow
2005-present	Tiffany Simms-Waldrip, M.D., Pediatric Resident
2005	Katrin Rhodes, rotating ACCESS graduate student
2006-present	Sam Kaneko, first year UCLA medical student (STTP)
2006-present	Kellie Lim, 4th year medical student mentor, UCLA Medical Specialties College Program
2006-present	Jenny Hernandez, graduate student (Pathology)
2006-present	Alan Ikeda, M.D., Pediatric Hematology-Oncology fellow
2006-present	Tara Lin, M.D., Adult Oncology, Postdoctoral fellow
2006	Andrew Goldsmith, ACCESS rotation student
2006-present	James Ch'ng, undergraduate student

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BOOK CHAPTERS

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2. Schmid I and **KM Sakamoto,** Analysis of DNA Content and Green Fluorescent Protein Expression. Current Protocols in Flow Cytometry, 7.16.1-7.16.10, 2001.

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4. Hagey A and **KM Sakamoto**. White Cell Disorders. Manuscript (online) for Textbook in Pediatrics, emedicine.com, 2001.

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ABSTRACTS

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14. Mignacca RC and **Sakamoto KM**. Transcriptional Regulation of the Human egr-1 gene by PIXY321 in a factor-dependent myeloid leukemic cell line. Presented to the American Association for Cancer Research meeting on Transcriptional Regulation of Cell Proliferation and Differentiation, Chatham, MA; October 1994.

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*66. Rodriguez-Gonzalez A, Kim KB, Crews CM, Deshaies RJ, and **KM Sakamoto**. Development of Protacs to target the estrogen receptor for ubiquitination and degradation in breast cancer cells. Accepted for an oral presentation. AACR meeting, Washington DC, April 2006.

67. Francisco Martinez F, Jimenez F, Machuca C, Villegas H, and **KM Sakamoto**. Transcriptional activation of krox-1 induced by sexual hormones in osteosarcoma cells. Accepted for a poster presentation. American Society for Gene Therapy Baltimore, Maryland. May 2006.

68. Cheng JC, Shankar D, and **KM Sakamoto**. Requirement of CREB in Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

69. Esparza SE, Shankar DB, and **KM Sakamoto**. Identification of Meis1 as a Target of CREB in Myeloid Leukemogenesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

70. Sandoval S, Shankar DB, and **KM Sakamoto**. Acceleration of Leukemogenesis in CREB Transgenic mice by Retroviral Insertional Mutagenesis. American Society of Hematology, Orlando FL, December 2006.

*oral presentation of abstract

INVITED PRESENTATIONS

1. **Sakamoto KM.** "Cytokine Signals and Cell Cycle Control During Myelopoiesis" Childhood Leukemia, Biological and Therapeutic Advances. April 17, 1998, Los Angeles, California.

2. **Sakamoto KM.** Serine/Threonine Phosphorylation in Cytokine Signaling Workshop sponsored by the National Cancer Institute. March 30, 1999, Washington, D.C.

3. **Sakamoto KM.** "Signal Transduction Pathways Activated by GM-CSF." October 29-30, 1999. ACS Professors Meeting, New York.

4.**Sakamoto KM.** "Signal Transduction and Cell Cycle Control in Myeloid Cells" for Meet-the-Experts Breakfast, American Society of Hematology, December 5, 1999, New Orleans, LA.

5. **Sakamoto KM.** CapCURE meeting, September 2000, Lake Tahoe. "Novel Approach to treat Prostate Cancer"

6. **KM Sakamoto and RJ Deshaies.** What SCF Ubiquitin Ligases Are and how they can be used to regulate cancer progression, 4/01

7. **Sakamoto KM**. Bone marrow cells regenerate infracted myocardium, Journal Club, 4/01

8. **Sakamoto KM.** Acute Leukemia for Pediatric Residents at UCLA School of Medicine, 7/01

9. **Sakamoto KM.** ITP, Olive View Grand Rounds, 8/01

10. **Sakamoto KM.** Childhood Leukemia: causes and treatment. American Cancer Society, Los Angeles Chapter, 10/01

11. **Sakamoto KM.** "The Role of SCF Ubiquitin Ligase in Human Disease: Implications for Therapy." Caltech Biolunch, March 6, 2002.

12. **Sakamoto KM**. "Targeting Cancer-Promoting Proteins for Ubiquitination and Degradation" Signal Transduction Program Area Seminar, Jonsson Comprehensive Cancer Center, UCLA. August 1, 2003.

13. **Sakamoto KM.** "Development of Approaches to Target Proteins for Ubiquitination and Degradation in Human Disease." Thesis Defense, Caltech. December 18, 2003.

14. **Sakamoto KM.** "Role of CREB in Human Leukemias." Gene Medicine Seminar Series. Jan 26, 2004.

15. **Sakamoto KM**. "Childhood Neutropenias." Pediatric Resident Noon conference. February 4, 2004.

16**. Sakamoto KM**, "The Role of CREB in human leukemias", Gene Medicine Research Seminar, January 26th 2004.

17. **Sakamoto, KM**. "CREB and Acute Myeloid Leukemia," Leukemia Research Group Meeting, March 4, 2004.

18. **Sakamoto, KM.** "The Role of CREB in Leukemogenesis," Pediatric Research Seminar, May 20, 2004.

19. **Sakamoto, KM**. Meet the Professors lunch for UCLA ACCESS graduate students. October 6, 2004.

20. **Sakamoto KM**. UCLA Training Program in Tumor Biology. Retreat for UCLA ACCESS graduate students. October 31, 2004.

21. **Sakamoto KM**. "Hematology Jeopardy" Pediatric Noon Seminar, December 13, 2004.

21. **Sakamoto KM**. "Transcriptional Regulators in Normal and Malignant Hematopoiesis," MBI Noon Seminar, November 30, 2004.

22. **Sakamoto KM**. "Targeting Proteins for Ubiquitination and Degradation in Prostate Cancer" GU SPORE seminar, December 21, 2004.

23. **Sakamoto KM**. "Writing your first NIH grant: an overview," Pediatric Research Seminar, April 7, 2005.

24. **Sakamoto KM**. "Targeting the Ubiquitin-Proteasome System for Cancer Therapy." Minisymposium on Modulation of Protein Stability, AACR, Anaheim, CA, April 20, 2005.

25. **Sakamoto KM**. "The Role of CREB in Myelopoiesis." Myeloid Workshop, Annapolis, MD, 2005

26. **Sakamoto KM**. "The Use of RNA Interference to Study and Treat Human Disease." Organizer, Cell Biology Methods workshop, PAS/SPR meeting, Washington, D.C., 2005.

27. **Sakamoto KM**. Young Investigators Workshop. American Society of Pediatric Hematology-Oncology meeting, Washington D.C. 2005.

29. **Sakamoto KM**. "Update on Acute Leukemia: Where we've been and where we are today." Pediatric Grand Rounds, Children's Hospital of Los Angeles. August 19, 2005.

30. **Sakamoto KM.** "Acute Myeloid Leukemia," Pediatric Resident Rounds, September 21, 2005.

30. **Sakamoto KM and Krasne S**. "Grants Writing Workshop," ACCESS graduate students, September 26, 2005.

31. **Sakamoto KM.** "COG Spring Meeting Report on Acute Leukemia." Tuesday 3pm Division Conference, March 28, 2006.

32. **Sakamoto KM.** "Successes and Challenges of Childhood Cancer: Leukemia as a Model." Life after Childhood Cancer, March 29, UCLA symposium sponsored by the Leukemia and Lymphoma Society of America.

33. **Sakamoto KM.** "The Cancer Problem." M294 Basic Concepts in Oncology Course, April 3, 2006.

34. **Sakamoto KM.** "Update on Childhood Leukemia." Pediatric Grand Rounds, Martin Luther King Junior Medical Center.

35. **Sakamoto KM.** "Choosing a Career in Basic Science Research." Young Investigator Workshop (organizer). ASPHO/SPR meeting. April 30, 2006.

36. **Sakamoto KM.** "RNA interference and Stem Cells," New Approaches in Stem Cell Technologies Workshop, SPR meeting, San Francisco, California. April 29, 2006.

37. **Sakamoto KM.** "Leukemia." M294 Basic Concepts in Oncology Course, May 10, 2006.

38**. Sakamoto KM**. " Update on Childhood Leukemias." Olive View Grand Rounds, May 17, 2006.

39. **Sakamoto KM.** "Leukemia" Pediatric Resident Noon Lecture, June 5, 2006.

40. **Sakamoto KM**. "State of Art lecture on AML/Myelodysplastic Syndromes" for fellows, Division Conference, Tuesday July 18, 2006.

41. **Sakamoto KM**. "Preparation of the RRC Site Visit." Division Conference, August 29, 2006.

42. **Sakamoto KM.** "Molecular and Cellular Characterization of MPD: The Role of CREB in Myelopoiesis." NIH/NHLBI grantees meeting on MPD and MDS, November 9, 2006.

A Meeting Report on the workshop, "New Technologies in Stem Cell Research" presented at the Society for Pediatric Research, April 29, 2006. San Francisco, California. In press.

RNA Interference and Stem Cells

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ABSTRACT

RNA interference (RNAi) is a powerful tool to study gene function, especially in stem cells. Small interfering RNAs (siRNAs) can effectively be introduced either with a vehicle or through viral vectors to transiently or stably inhibit the expression of a particular gene target. Much is known about the optimization of siRNAs and method of delivery in mammalian cells. In this review, we discuss design considerations for siRNAs, methods of delivery, optimization of siRNAs, applications to study genes in stem cells, therapeutic applications, and remaining hurdles. With recent advances in RNAi, it is likely that application of this technology will increase in the future.

INTRODUCTION

RNA interference (RNAi) describes the inhibition of gene expression by double-stranded RNAs (dsRNAs) developed in the mid 1990's [1]. Guo and Kemphues discovered that sense RNA was as effective as antisense RNA for suppressing gene expression in nematode worms (*Caenorhabditis elegans*) [2]. This was followed by the introduction of dsRNA into worms. When single-stranded antisense RNA and double stranded RNA was introduced into worms, they found that dsRNA was more effective than either strand individual in downregulating genes [1].

RNAi is a multi-step process that involves the generation of small interfering RNAs (siRNAs) *in vivo* through the activity of the RNase III endonuclease 'Dicer." The resulting 21- to 23-nucleotide (nt) siRNAs mediate degradation of their complementary RNA [3]. It is now thought that RNAi induces gene silencing through various mechanisms. One is by sequence-specific targeted gene silencing. The second is through translational repression (microRNAs). Finally, it has been reported that RNAi maintains silenced regions of chromosomes [3].

Basic mechanisms of RNAi

Long double-stranded RNAs (dsRNAs) are the precursors of the siRNAs that trigger the RNAi effect. When dsRNAs enter cells, they are cleaved by an RNase III-like enzyme known as Dicer into siRNAs (Fig 1). These 21-23 nt siRNAs forms part of a siRNA- protein complex known as RISC (RNA-induced silencing complex), which contains helicase activity that unwinds the two strands

of RNA molecules, allowing the antisense strand to bind the targeted RNA [4-7]. RISC also has endonuclease activity that hydrolyzes the target RNA at the site where it binds the antisense strands. Formation of RISC is critical for mRNA degradation. Therefore, the RISC complex mediates the sequence-specific degradation of the target RNAs that contain homologous sequences to the siRNA.

What is a desirable target for RNAi?

Desirable targets of RNAi include genes that are amplified or overexpressed in cells leading to a specific phenotype. The other is an aberrant protein that is encoded by a dominant mutant allele. Examples include oncogenes that produce transformation in mammalian cells. However, genes that are abundantly expressed or have a prolonged half-life may not be efficiently inhibited. Similarly, genes that are redundant may not be effectively downregulated.

The advantages of RNAi are that the targeted degradation is very specific and can result in variable levels of downregulation such that gene dosage effects can be studied. This technology is much easier, quicker, and less expensive than generating knockout mice. RNAi can also be used to inhibit expression of multiple genes at the same time [8-10].

Design of siRNA

The use of siRNAs has become a common method of downregulating gene expression to screen gene function in many cell types, including stem cells.

Although long dsRNAs (>30 nt) are effective in suppressing gene expression in plants, Drosophila, and C. elegans; long dsRNAs are cleaved by Dicer to form siRNAs when introduced into mammalian cells and these siRNAs lead to mRNA degradation. However, in mammalian cells, long dsRNAs activate the interferon response pathway leading to non-specific mRNA degradation. The dsRNA-dependent protein kinase (PKR) is activated, resulting in nonspecific translational inhibition [11, 12]. Therefore, the usefulness of dsRNA in mammalian cells is limited.

In general, 21-23nt siRNAs are too short to activate the non-specific dsRNA response pathway, but are effective in inhibiting the expression of specific targets. There are several limitations of using this technology in mammalian cells. In fungi, plants, and worms, siRNAs can be replicated *in vivo*. In mammalian cells, siRNAs do not prime the synthesis of dsRNA to form addition siRNAs, which may explain why this technology is less effective [9]. Nevertheless, there are several examples in which siRNAs are effective in a variety of mammalian cell types, including stem and progenitor cells [1, 13].

Optimization of siRNAs in mammalian cells is dependent on several factors. One is the accessibility of the target sequence to the desired mRNA substrate. Previous reports have suggested that selecting a target sequence 100-200 nts away from the translational initiation sequence AUG of the gene is desired [1]. However, successful inhibition of gene expression has also been reported for siRNAs targeting various sequences, including the 3' untranslated region [14]. Targeting of the 3'UTR is also useful if rescue experiments are to be performed. There is no reliable way to predict or identify the ideal sequence for

siRNA. Several reports have suggested that sequences that form the stems of the hairpin siRNAs, the loop size and the sequences at the base of the loop might also affect siRNA-induced gene inhibition. Other determinants include thermodynamic stability; siRNA with lower thermodynamic stability for base pairing at the 5' end of antisense (guide) strand and in the middle of the siRNA were more effective at RNAi than those that had stronger base pairings in these regions due to affects on uptake of guide strand into RISC and enhancing RISC binding to target mRNA.

The sequence of siRNAs should be carefully designed. The number of nucleotides should be between 19-23 nt. The GC content should be between 30 and 50%. The preferred format should be $AAN_{19}TT$. Sequence specificity to at least two nucleotides should be confirmed by Blast comparison of the NCBI GenBank database. Finally, one should query against the SNP database [10].

Optimization of siRNA

To ensure that the gene of interest is effectively downregulated by the siRNA, it is now recommended that at least three different siRNA sequences per target be designed [15, 16]. More robust knockdown of genes has been reported using this approach of creating "multiplicity" controls. Inhibition of expression has been reported for up to 5 to 10 days when using "pools" of siRNAs in transfected cells.

siRNA concentrations must also be optimized. In general, concentrations of siRNAs greater than 100 nM are considered to be toxic. Various amounts of siRNAs should be tested for each specific cell type. This should be considered when one is using multiple siRNA sequences. Multiple cell lines should also be

tested to validate response and downregulation. Finally, a nucleotide Blast search should be performed to determine whether the siRNA sequence would target another gene. In terms of controls, scrambled or mutated sequence (www.sirnawizard.com), and unrelated genes, e.g. luciferase are commonly used. To validate successful downregulation of the target gene, it is recommended that a Western blot analysis be performed to assess protein levels and Northern blot analysis or RT-PCR to measure RNA levels. Demonstration of lower mRNA levels is critical to rule out a microRNA effect and translational inhibition of gene expression. To control for off target effects, one can measure interferon response genes, including *OAS1*, *OAS2*, and *INFB1* by RT-PCR[1].

Delivery of siRNA to cells

In mammalian cells, efficiency of siRNA to cells transiently depends on the vehicle or mode of delivery and the cell types. Approaches to introduce siRNAs into cells include a lipid-based vehicle, e.g. lipofectamine, or non-lipid based approach, e.g. calcium phosphate, electroporation. The disadvantages of this approach are that the siRNAs are non-renewable and are only effective as long as they are bath-applied to cells. An alternative strategy has been to deliver siRNAs through a DNA-vector-mediated RNAi approach.

Due to the transient nature of gene silencing produced by oligonucleotide siRNAs and their high costs of chemical synthesis, alternative approaches to introduce siRNAs in plasmid vectors have been developed. A variety of expression vectors are now available. Expression is driven by either the U6 (small nuclear RNA) or H1 RNA polymerase III promoters to drive expression of

sequence-specific short hairpin RNAs (shRNAs) in mammalian cells [2]. These systems are based on the expression of siRNAs either as two separate strands or as a single shRNA. It is thought that the shRNAs are processed by Dicer to active siRNAs *in vivo* [17-19].

For stable expression in stem cells, the successful delivery has been demonstrated with viral vectors. Various recombinant viral vectors have been developed to deliver shRNAs in mammalian cells [10, 20]. Lentiviral vectors are especially effective. The reasons for this are that lentiviruses have broader tropism and receptor independent delivery. They also have the ability to integrate into the genome for stable gene silencing. Finally, lentiviral transduction and expression of shRNAs do not require cell division for integration into the genome [21]. Lentiviral transduction has been successfully performed in cell lines, mouse hematopoietic stem cells (HSCs), and ES cells [22-24].

Adenoviral vectors have also been reported to be useful for delivering siRNAs to target cells. This vector system has been used to downregulate genes in liver. However, this vector system has limited utility in stem cells, since low transduction rates have been found in embryonic stem cells (ES) and HSCs. This is most likely due to the fact that the receptor for adenovirus is not highly expressed in stem cells [25]. Similarly, adenoviral-associate vectors (AAV) have been successfully used to deliver RNAi to non-stem cells [1].

If the stable transfection or transduction of siRNAs results in toxic effects to cells, an alternative approach is to use the inducible expression of shRNAs. The tetracycline/doxycycline regulated form of U6 or H1 promoter has been successfully used. If there is leakiness, other inducible systems such as an

Ecdysone-inducible system, are more tightly regulated with less background. A newer approach has been CRE-lox inducible system[26]. Most recently a doxycycline inducible vector that contains a KRAB domain from one third of zinc finger domains was used in cell lines, mouse ES cells, epithelial breast cancer cells, rat brains, CD34+ cells, and in transgenic mice [27].

Application of RNAi in Stem Cells

There is now emerging evidence that RNAi can be used to study gene function and for therapeutic application. ES cells are pluripotent stem cells that are derived from the inner cell mass of the 3.5-day-old mouse blastocyst [1, 28]. These cells are desirable models to study the regulation of development and cell lineage commitment and differentiation, since ES cells can give rise to all three germ layers. This system is a powerful tool to study development.

Interestingly, long dsRNA has been used in ES cells, but only when undifferentiated. The reason for this is unknown. In differentiated ES cells, siRNAs have been found to be effective in inhibiting genes such as PU1 and c-EBPa [1]. A variety of other genes have been downregulated in ES cells, such as Shp-2 and Oct-4. Synthetic shRNAs recently have been shown to be efficiently transfected transiently with lipofectamine [29]. More commonly, viral vector systems have been used to transduce genes of interest for stable expression of shRNAs.

HSCs are a self-renewing population of cells in the bone marrow that give rise to all differentiated hematopoietic cells [1]. A number of genes have been targeted using RNAi in HSCs. Growth factor receptor genes, clusters of

differentiation, chemokines, oncogenes (*bcr-abl*), tumor suppressors, HIV genes, globin genes, and RPS19 expression have all been successfully targeted. In most cases, retroviral or lentiviral vector systems were used. Electroporation has been used successfully to introduce dsRNA in HSCs [13]. Lipofectamine has also been reported to effectively transfect oligonucleotide siRNAs into hematopoietic progenitor cells [30]. HSCs that are transduced with shRNAs can then be studied *in vitro* using methylcellulose colony assays or *in vivo* in bone marrow transplantation experiments.

NSCs and MSCs

Neural stem cells (NSCs) have also been transduced with shRNAs to downregulate genes. Examples of genes inhibited in NSCs by RNAi are MELK, PPARγ and B27.a genes [31-33]. Mesenchymal stem cells (MSCs) have been studied using both viral and non-viral methods. Genes inhibited using viral vectors were beta-catenin, Msx2, and mecdin [2, 34]. Non-viral liposomal methods to introduce siRNAs into MSCs have been used to inhibit EGFR and Connective Tissue Growth Factor [35, 36]. Recently, a Transfection Microarray Approach was generated in which siRNAs were applied onto slides that are coated with poly-L-lysine and fibronectin. MSCs were then placed on top of the poly-L-lysine and siRNA "sandwich." Fluorescent microscopy was used to then visualize and quantify the degree of downregulation [37, 38]. A similar approach was used with HeLa cells placed on slides treated with siRNAs, in which cells were then followed "real time" using time-lapse fluorescent microscopy as a high throughput method to screen for genes involved in chromosomal segregation [39].

shRNA libraries

One of the technological advances in the RNAi field has been the development of shRNA libraries to screen for genes that regulate a specific pathway or biological function. Many of the libraries rely on lentiviral vector-based expression. Libraries have been used to identify deubiquitinating enzymes [40], sensitivity to small molecule inhibitors, novel cancer genes, and previously unidentified components of signaling pathways. A recent report from the Broad/MIT group (The RNAi consortium) used an shRNA library with 72,600 clones targeting 10,500 human and 5,300 mouse genes. It is anticipated that the numbers of genes targeted could be as high as 15,000 human or mouse genes. Viruses expressing shRNAs can be transiently or stably transduced into mammalian cells [41]. Genes that are involved in a particular cellular process will be identified through identification of the shRNA clones that block the function of the gene. An inducible shRNA library has also been used recently to identify genes that regulate proliferation or survival of diffuse large B cell lymphoma cells to seek novel targets for therapy [42].

Therapeutic applications of RNAi

The field of RNAi is advancing at a rapid pace. The application of RNAi as gene therapy is now being realized. In mice, delivery of siRNA to downregulate Fas by hydrodynamic tail injected resulted in protection from fulminant hepatitis [43]. A recent report by Samakoglu et al. has demonstrated that sickle globin gene can be downregulated in CD34+ cells using a lentiviral shRNA, with a concomitant increase in g-globin expression in erythroid specific manner [44]. Another advance

has been the successful RNAi-mediated gene silencing in non-human primates. The first report of systemic delivery of *APOB* siRNA in non-rodent species was recently reported [45]. APOB is a component of LDL and regulates the storage and metabolism of cholesterol. A liposomal formulation of *APO-B* siRNAs was intravenously administered into cynomologous monkeys with effective inhibition of APOB levels after 48 hours and 11 days. Plasma levels demonstrated that not only LDL and cholesterol levels were lower than controls but HDL levels were not affected. Although previous success was shown with hydrodynamic tail injection of oligonucleotide siRNAs in rodents, this was the first report of siRNAs successfully targeting a gene in non-rodent models.

Remaining challenges

Although the field of RNAi has rapidly progressed, there are several hurdles that remain before fully applying this technology in humans. The specificity and toxicity of siRNAs must be more rigorously examined. The use of lentiviral vectors in gene therapy has led to insertional mutagenesis and malignancies, which must be overcome. Newer generations of lentiviral vectors are currently being studied. Stability of siRNAs is also problematic for long-term use. However, recent advances in nanotechnology have demonstrated that delivery of siRNAs using nanoparticles has potential in the clinics [46]. Given the advances in the field, it is highly likely that within the next few years, RNAi will become a viable approach to treat human disease.

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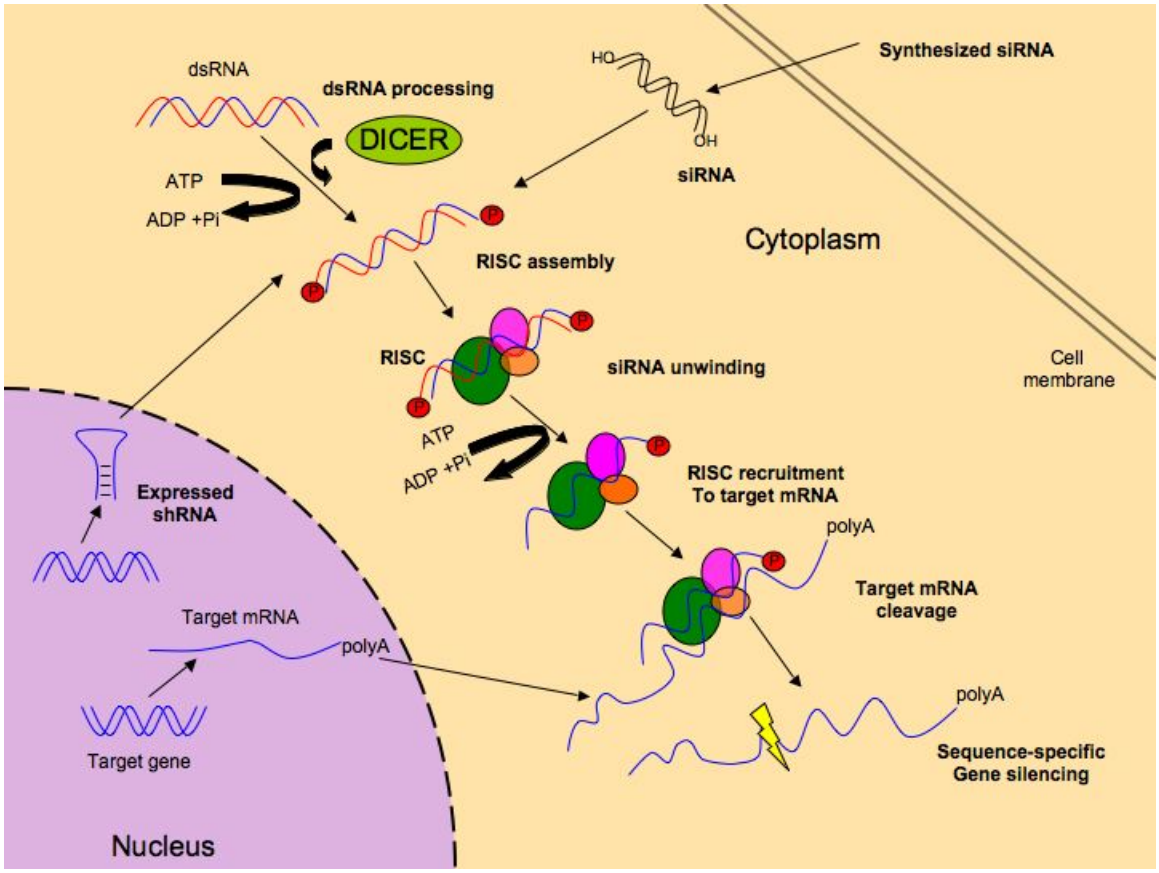

Figure 1. siRNA pathways that target mRNA for degradation.